

EFFECTS OF CAFFEINE AND WARRIOR STRESS ON BEHAVIORAL HEALTH:  
AN ANIMAL MODEL

by

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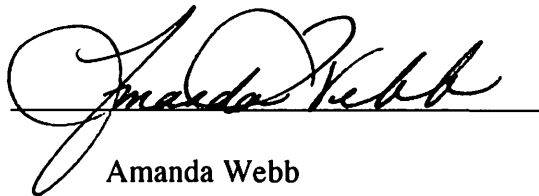
## **DEDICATION**

This project is dedicated to my husband, Robert Webb, whose service to our nation and love of caffeine were the inspiration for this work.

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## **ABSTRACT**

Title of Thesis: Effects of Caffeine and Warrior Stress on Behavioral Health: An Animal Model

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Caffeine is commonly consumed by military service members with upwards of 45% regularly ingesting caffeine in amounts exceeding 300 mg per day. Some literature highlights positive effects of moderate caffeine consumption; other studies suggest that >300 mg/day can result in negative behavioral health outcomes. Using an animal model (N=32), the present study examined effects of caffeine and stress on behaviors related to anxiety and depression using a full factorial mixed design: 2 (no caffeine, caffeine) x 2 (no warrior stress, stress) x 3 (baseline, 7 days, 14 days). Caffeine animals were chronically exposed to a caffeine-sucrose solution (1 g/L in 7% sucrose solution) via home cage water bottles and acutely exposed to a caffeine-sucrose solution (60 mg/kg in 7% sucrose solution) via a feeding syringe 30 minutes prior to stress. Stress was administered in two phases. The first 7-day stress iteration employed the Warrior Stress Paradigm (WSP). The second 7-day stress iteration added a sleep disruption component. It was hypothesized that the combination of caffeine and stress would result in increased anxiety-like and depressive-like behaviors in exposed animals. Horizontal activity was examined to measure general health and locomotion; ratio of center time assessed anxiety-like behaviors; and vertical activity assessed depressive-like behaviors. Caffeine

was significantly associated with higher rates of depressive-like behaviors in rats. This finding is in accordance with studies performed with human subjects – where moderate to high levels of caffeine appear to be associated with depression.

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## **CHAPTER 1: Introduction**

Physical, emotional, and psychological wounds are among the most debilitating and costly burdens of war (32). The tolls of war can afflict families and communities for several generations; however, the warfighter bears the deepest burdens (32). In the wake of ongoing military operations, there is an increased interest in military behavioral health and scholars are engaging in scientific research aimed at uncovering the etiology and the most effective treatments for a variety of disorders (11; 37; 44; 93). Current estimates suggest that there have been approximately 177,461 new cases of Posttraumatic Stress Disorder (PTSD) across all services since 2000 (24). Additionally, Lapierre et al. (44) found that 44% of returning Iraq and Afghanistan veterans reported depressive and posttraumatic symptoms. These estimates are particularly concerning given the long term financial, occupational, marital, psychiatric, and healthcare utilization costs associated with these syndromes (42; 88). In light of the high behavioral health problems reported by returning veterans, it is becoming increasingly important to unravel the factors that are contributing to the development and maintenance of behavioral health disorders (24; 44). Caffeine and stress are two of those potential factors.

A variety of factors, including stress and chronic sleep disruption contribute to the behavioral health problems reported by service members (9; 46; 56; 64). One countermeasure used by military personnel to reduce the effects of stress, fatigue, and low mood is caffeine consumption (10; 39; 82; 94). The use of caffeine in the military as a performance-enhancing supplement is a controversial topic (90). Although there is an abundance of research on the positive effects of caffeine as a fatigue countermeasure and

performance enhancer (29; 85; 94), little research exists in the combined effects of caffeine, sleep disruption, and chronic stress on behavioral health (102).

To address this research gap, the present experiment examined effects of caffeine consumption, sleep disruption, and stress on indices of behavioral health in rats. Several ethical and logistical issues prevent the use of humans in true controlled experiments that manipulate stress and caffeine exposure, so an animal model was used instead. Animal models serve as proxies for human research and allow for controlled experiments. The following sections provide an overview of: 1) caffeine use in the general population and in the military, 2) stress, 3) sleep disruption, 4) anxiety and PTSD, and 5) depression.

## **CAFFEINE**

Caffeine is the most widely consumed psychoactive substance worldwide (77; 86; 87). According to recent estimates, more than 85% of children and adults consume caffeine regularly (3; 46; 86). Moderate caffeine consumption (i.e., 250 mg or less), typically in the form of food (e.g., chocolate) and drinks (e.g., coffee, tea, energy drinks, and soft drinks), improves attention and performance (28; 77; 85). Additionally, caffeine has been found to improve psychomotor functioning, mood, cognitive functioning, wakefulness, and athletic performance (39; 85).

In addition to the positive benefits of caffeine use, there also is an abundance of research highlighting the negative effects of consumption in excess of 300 mg, commonly referred to as “caffeinism” (85; 86). Caffeinism is described by the American Psychiatric Association (3) as caffeine intoxication and includes notable symptoms such as: restlessness, nervousness, excitement, insomnia, and psychomotor agitation. These signs and symptoms are responses to a recent ingestion of caffeine, typically in doses

exceeding 250 mg (3). This psychophysiological response is similar to that of anxiety and research indicates that high doses of caffeine may lead to anxiety (85; 86).

A notable effect of caffeinism is the increase in anxiety and vigilance for social threats (85; 86). Researchers assessing the effects of caffeine on the brain regions associated with social threat processing and anxiety concluded that caffeine increases threat-related activation in the midbrain (86). They also highlighted that other brain mechanisms involved in this process are linked to clinical anxiety disorders, especially PTSD – a mental health condition of particular concern in the military.

### **Caffeine in the military**

In the military, service members consume greater amounts of caffeine than do their age-matched civilians (39; 46; 82). Lieberman et al. (46) reported that 82% of soldiers in the U.S. Army consumed at least one caffeinated beverage a week with daily consumers averaging 347 mg/day. Coffee was the primary source of caffeine, though younger male soldiers preferred energy drinks (46). A recent study by the Centers for Disease Control and Prevention (12) of almost 1,000 deployed service members found that approximately 45% consumed energy drinks. Caffeine content in energy drinks ranges from 50 to more than 500 mg per drink (73). The high caffeine content (e.g., each energy drink contains the caffeine equivalent of one to three cups of coffee) offers service members a quick solution to their fatigue (12).

Deployed service members typically use caffeine in excess of recommended dosages to counteract the negative effects of chronic sleep disruption (12; 41; 46). High consumption percentages also may be the result of perceived effectiveness of the drug and the accessibility of energy drinks on military bases and in forward deployed

environments (12; 39). Given the austere environmental conditions and high rates of sleep disruption among military personnel, service members may be at increased risk for caffeinism. Caffeine increases blood pressure, nervousness, tension, anxiety, vigilance for social threats, and arousal (25; 85; 86). Experiencing this state of hypervigilance, hypersensitivity, and arousal prior to a traumatic event may serve to potentiate the symptoms of anxiety or symptoms of PTSD. At the same time, exposure to the traumatic event places the individual in a heightened state of arousal. Therefore, consumption of caffeine shortly before, during, or post-stressor may amplify psychophysiological symptoms and perpetuate this hyperaroused state (86). Given that service members often attempt to counter the negative effects of chronic stress and sleep disruption by consuming caffeine, further examination of this relationship is warranted.

## **STRESS**

Stress occurs when internal or external events disrupt an organism's homeostasis and exceed the adaptive capacity of the organism (22; 33). The stress-response can manifest as either eustress or distress. Eustress is an organism's stress response to positive stimuli and distress is an organism's stress response to negative stimuli (33). Stress can be acute or chronic. Acute stress is characterized as an extreme and uncommon situation that results in distress (17). Acute stress is short in duration (17), but can be experienced repeatedly (e.g., on a daily basis) (26). Chronic stress is long term, can be continuous, and is likely to be encountered on a daily basis (17).

Stressors can be organized into six principal categories: 1) psychological, 2) biological, 3) environmental, 4) physical, 5) economic and, 6) occupational. Psychological stressors include a variety of constructs such as perceived versus actual



control, and cognitive appraisals that affect the stress response. Biological stressors are any stimuli (e.g., low blood sugar and heat stroke) that disrupt an organism's ability to maintain homeostasis (22). Environmental stressors include conditions such as extreme temperatures, high altitude, and/or sand storms. Physical stressors are events or conditions that tax physical capabilities. For example, some military service members are required to conduct 20 mile forced ruck marches with 70 pounds of gear. Other examples of physical stressors include airborne operations and high altitude, low-opening (HALO) operations. Economic stressors include situations such as unemployment and low income. Occupational stressors are work-place events that increase stress (e.g., internal politics, deployments, layoffs, cutbacks, and/or overtime).

There is a large body of literature indicating that stress can result in increased vulnerability to physical and psychological disease or death (7; 17; 96). Examples of major negative health effects include: coronary heart disease, immune suppression, fatigue, depression, disturbed sleep, gastrointestinal disturbances, decreased libido, anxiety/panic-like symptoms, and increased physical injury (13; 16; 99). Military service members are at increased risk for the development of a host of physical and psychological diseases because of their high rate of exposure to acute, repeated acute, and chronic stress.

### **Warrior stress**

Military service members encounter stressors such as: exposure to combat, frequent deployments, unpredictable schedules, poor leadership, high-risk duties, frequent relocations, separation from family, sense of isolation, rigid behavioral standards, low pay, physical wounds (e.g., loss of limb), and other individual and family

stressors (50). In a study examining chronic stress in Ranger School, Bernton et al. (7) found alterations in candidates' endocrine and immune function. Henning et al. (36) found that military operational stressors decrease anabolic hormones, skeletal muscle mass, and bone mineral density. Additionally, the number of service members reporting behavioral health problems is concerning (24; 37; 44). Hoge et al. (38) indicated that approximately 17% of Soldiers and Marines returning from combat operations screened positive for PTSD, generalized anxiety disorder (GAD), and depression. Given the relationship between stress and behavioral health disorders, it is necessary to understand what role caffeine may play in the development or maintenance of behavioral problems. There are ethical issues associated with exposing humans to high caffeine doses and warrior stress. Animal studies help circumvent the ethical limitations inherent in human research and provide a research strategy to examine the combined effects of stress and caffeine on indices of behavioral health.

## **SLEEP DISRUPTION**

Sleep disturbance is a significant problem affecting deployed military personnel (10; 64). Acute and chronic stress, shift work, high operational tempos, demanding physical activities, and poor nutrition all disturb sleep quality (10; 64; 95). Sleep disruption can be especially problematic for military service members with high-risk missions (e.g., pilots, special forces) (10; 46; 94). As noted by Caldwell and Caldwell (10), a single mistake by a pilot can cost millions of dollars in damages. According to the Department of Defense Office of the Deputy Assistant Secretary of Defense (19), less than 40.9% of active duty personnel report getting 7 to 8 hours of sleep nightly. Peterson et al. (64) found that approximately 75% of deployed Air Force personnel reported their

sleep during deployment was significantly worse than their sleep when not deployed. Further, 86.5% reported getting only 4.5 hours of sleep a night, the minimum sleep required for optimal performance in sustained or prolonged deployed operations (64).

The adverse effects of poor sleep are well documented. Sleep disruption can lead to decreased cognitive functioning and decreased alertness (102), lapses in attention, impaired working memory, decreased visual perception, increased susceptibility to accidents, impaired psychomotor functioning, poor decision making, decreased motivation, increased risk-taking behavior (49; 103), and impaired marksmanship (94). Caldwell and Caldwell (10) highlight that 2 to 3 days of inadequate sleep can result in a near total loss of operational readiness among aviation units. One psycho-pharmaceutical intervention intended to combat the adverse effects of sleep disruption among military personnel is caffeine (29; 94).

### **Caffeine and sleep disruption**

A number of studies highlight the effectiveness of caffeine as a means to counter the unfavorable effects of sleep disruption. Wesensten et al. (102) found that 600 mg of caffeine significantly improved performance and alertness in a sample of 50, sleep deprived (for 54.5 hours), adult males. Similarly, Tharion et al. (94) found that 200 to 300 mg of caffeine enabled 72-hour, sleep-deprived Special Operations Forces Navy SEALs (Sea-Air-Land) to maintain marksmanship accuracy while increasing speed of trigger pull and target sighting. In a study of Navy SEAL trainees with 72-hour sleep deprivation, Lieberman et al. (47) concluded that 200 and 300 mg of caffeine significantly improved cognitive performance.

While the research literature provides compelling evidence that moderate caffeine doses can counter the negative effects of sleep deprivation, emerging literature is also showing that caffeine can disrupt sleep by decreasing total sleep time (101). Additionally, chronic caffeine consumption may exacerbate sleep problems by interfering with service members' abilities to initiate sleep, a sleep variable consistently indicative of long-term sleep problems, such as insomnia (64). The combined effects of sleep disruption, warrior stress, and caffeine remain unknown. An animal experiment to address this research gap is presented.

## **ANXIETY AND PTSD**

Anxiety disorders are syndromes that elicit clinically relevant behavioral disturbances in response to fear or anxiety. These behavioral disturbances manifest as physiological, cognitive, and psychological fear responses and are considered excessive and out of proportion to the stimuli (3; 40). There are a variety of ways that anxiety can manifest making it one of the most prevalent behavioral health disorders in the United States (75; 83). In fact, lifetime estimates for anxiety disorders are 28.8% with medical expenditures ranging from \$42 to \$47 billion annually (75; 83). These costly individual and societal tolls underscore the importance of identifying factors that contribute to the development of anxiety disorders.

Although two distinct concepts, PTSD and anxiety are characteristically similar in important ways. For example, fear and anxiety-related behaviors are the essential characteristics of anxiety disorders and are similar to the arousal and avoidance responses evidenced by individuals exposed to fearful stimuli (3; 21; 40). Jones and Barlow (40) elaborate on this relationship and describe anxiety as a feedback loop whereby anxiety-

related symptoms manifest as chronic overarousal resulting in distorted information processing. Ultimately, this cycle results in hypervigilance and can result in a state of anxious apprehension (40). As described, the symptom overlap between PTSD and anxiety is considerable, providing increased opportunities to study these complex disorders.

Caffeine mimics the symptoms of anxiety and may play a key role in the manifestation of these psychophysiological responses (85). Given the high prevalence rate of caffeine use in the military and service members' exposure to chronic sleep disruption and stress, it is critical to understand the relationship between chronic caffeine use and behavioral health.

## **DEPRESSION**

A study by Lapierre et al. (44) found that approximately 44% of veterans returning from deployment report clinical levels of depression and PTSD. Further, across all service branches, approximately 10% of active duty personnel report high levels of depression (18). The American Psychiatric Association recently acknowledged that some individuals exposed to trauma may develop symptoms characteristically similar to depression (e.g., dysphoria and anhedonia), and not anxiety or fear-based symptoms (3). Due to differential symptom development, PTSD and similar trauma disorders were moved out of the Anxiety section and into a separate section of the DSM-5 entitled *Trauma- and stressor-related disorders* (3). Individuals suffering from PTSD may experience symptoms characteristically similar to depression such as: persistent negative emotional state, markedly diminished interest or participation in significant activities,

persistent inability to experience positive emotions, and feelings of detachment or estrangement from others (3).

As described previously, caffeine may exacerbate symptoms of anxiety; however, the relationship between caffeine and depression is less clear (84; 98). Some studies suggest caffeine may protect against the development of depression (51; 60; 98), yet others show caffeine use as strongly associated with depression and other psychiatric disorders (27; 31; 76; 84; 92).

Human and animal studies indicate that caffeine may protect against the development of depression. A meta-analysis of observational studies by Wang et al. (98) concluded that coffee and caffeine consumption were significantly associated with reduced risk of depression. In a prospective study of women free of clinical depression at baseline, Lucas et al. (51) found that the risk of depression decreased as caffeinated coffee consumption increased, suggesting that caffeine may protect against the development of depression. A cross sectional study of middle-aged Finnish men conducted by Ruusunen et al. (79), also concluded that coffee was associated with a decreased risk of depression, though they found no association for tea and general caffeine intake. Finally, an animal study examining the long-term effects of caffeine and stress on depressive-like symptoms in rats concluded that caffeine may serve as an antidepressant (62).

Conversely, other literature reports that caffeine is associated with high rates of depression and psychiatric disorders. Among a sample of 83 hospitalized adult inpatients, Greden et al. (31) found that high caffeine consumers (i.e., at least 750 mg of caffeine per day) scored significantly higher on measures of anxiety and depression than

moderate or low caffeine consumers. In a study of college students, Gilliland and Andress (27) reported similar outcomes with moderate and high caffeine users scoring significantly higher on measures of depression and anxiety than did non-caffeine users. A case study of caffeine intoxication reported an association between high caffeine consumption, sleep deprivation, and a spontaneous suicide attempt by a non-suicidal, mentally healthy adult (92).

The literature exploring the relationship between caffeine and depression has produced mixed results. Additionally, the aforementioned studies did not examine the combined effects of caffeine, repeated stress, and sleep disruption on indices of behavioral health. Therefore, the present experiment examined the combined relationship of common military deployment stressors (e.g., repeated stress, sleep disruption, and caffeine intake) using an animal model.

### **IMPORTANCE OF ANIMAL MODELS**

The ethical issues associated with studying caffeine as a potentiator for behavioral health disorders precludes the use of humans as subjects in a true experiment that manipulates the independent variables and includes appropriate controls. However, research indicates that animals, including rodents, are an appropriate substitute to study anxiety-like and depressive-like behaviors (61; 105). Animals provide a unique opportunity to examine the individual and collective contributions of caffeine exposure and warrior-related stress to the development of anxiety and mood-related behaviors. Researchers can expose animals to acute and chronic controlled doses of caffeine, disrupt sleep cycles, and expose animals to stressors that mirror a military deployed environment. Examining these relationships under controlled conditions provides researchers an

opportunity to examine causal hypotheses regarding caffeine use, stress, and behavioral outcomes relevant to behavioral health.

### **Animal models of anxiety and depression**

Animal models are widely accepted as effective proxies for human research (15; 61; 71; 81; 91). Rodents, like humans, evidence anxiety-like and depressive-like behaviors when confronted with aversive stressful events such as exposure to predators. These symptoms become increasingly pronounced as the intensity of the stressor increases (89). Perry (63) reported that exposure to stress reliably increased stress hormones and produced behavioral changes indicative of depression in male Sprague-Dawley rats. Zoladz et al. (105) assessed general anxiety levels in rats after exposure to a cat (i.e., a predator exposure) and psychosocial stressors, such as social instability and chamber and tone exposure. Zoladz et al. (105) discovered that predator stress combined with psychosocial stressors resulted in increased hyperarousal and anxiety-like behaviors (e.g., startle and physiological reactions) in exposed rats. Such behavioral changes mirror those of individuals diagnosed with PTSD. Extant research using animal models of PTSD also highlights the similarities between anxiety-like fearful behaviors and avoidance (14; 105).

The open field activity (OFA) allows valid and reliable measures to evaluate anxiety-like and depressive-like behaviors in rats (71; 81). Environmental conditions that include open areas allow researchers to evaluate the natural behaviors of rodents by assessing activity and locomotion (71; 81; 89). These environments allow for the assessment of natural behaviors such as the rodent's exploration of new areas moderated by avoidance of new and potentially dangerous situations (57; 71; 81). Any disruptions



to the animal's natural behaviors are indicative of mood-symptoms such as anxiety or depression (14). For example, an animal evidencing anxiety-like behaviors will decrease the amount of time spent in the center of an open field in favor of time spent in corners and closed-off spaces (57; 71; 81). With regard to depression, animals evidencing depressive-like behaviors will show fewer rearing behaviors (i.e., standing on their hind legs). Rearing is an indicator that the animal is trying to escape and is considered a healthy and normal activity. Research based on the learned helplessness model indicates that low levels of escape behaviors are indicative of depression (78). While not an exact replica of human behaviors, animal models are useful to study symptoms of behavioral health disorders by measuring distinct behavioral responses. Therefore, the present experiment used an animal-model to examine effects of stress and caffeine on anxiety-like and depressive-like behaviors.

### **Animal models of caffeine**

Animal models of caffeine exposure use a variety of administration routes including: oral self-administration, intraperitoneal (IP) injection, subcutaneous (SC) injection, gavage, and implantation of osmotic pumps. Levels of caffeine exposure range from low to high. Studies examining chronic availability of caffeine use a 1 g/L caffeine solution in the animals' drinking water (59; 65). Pollard (67) identified 60 mg/kg caffeine as a high acute dose of caffeine when administered orally via a gavage. Animal models have reported that caffeine produces anxiety-like responses in rats (8; 59; 61). Additionally, in their work examining effects of long-term caffeine exposure on rats experiencing chronic unpredictable stress, Pechlivanova et al. (62) found that caffeine had antidepressant and anxiolytic effects.

### **Animal models of stress**

Animal studies have demonstrated that rodents exposed to stress evidence behaviors associated with anxiety, depression, and PTSD (14; 30; 59; 61; 62). Those responses are measured using open mazes and open environments, which assess the degree to which animals show disruptions to exploratory behavior (1; 14; 30). A variety of stressors including restraint stress, psychosocial stress (e.g., housing instability), environmental stress (e.g., heat and cold), food restriction, and shock are used to examine the effects of acute, chronic, and unpredictable stress (52; 59; 66; 105). The behavioral and physiological responses evidenced by rodents after exposure to a predator (or predator scent; e.g., a fox) are characteristically similar to those experienced by service members who are exposed to enemy combatants (2; 14; 15; 30). Similarly, chronic variable stress paradigms (e.g., Warrior Stress Paradigm [WSP] – see Methods section for description) replicate environmental stressors comparable to a combat environment. As such, they are considered the closest approximation to military stress conditions (30). The present experiment used the WSP to replicate predator and unpredictable sensory stressors similar to a combat environment under controlled conditions in an animal laboratory.

### **Animal models of sleep disruption**

Military service members serving in a deployed setting frequently encounter environmental noises during their sleep cycle. Animal studies of sleep disruption employ a combination of environmental noises to disrupt sleep (63; 70). In his examination of acute and recurrent stress during adolescence and its subsequent effect on adult behavioral health, Perry (63) used intermittent high and low frequency environmental

noises to disrupt the sleep cycle in rats. Noises included loud banging, bells, voices, shattering glass, and vehicular traffic, all of which are similar to military combat environments. The present experiment used a similar sleep disruption technique and exposed animals in the stress condition to a range of high and low frequency environmental noises.

## CHAPTER 2: Overview and Specific Aims

### OVERVIEW AND SPECIFIC AIMS

The potential relationship between caffeine and the development of behavioral health problems after exposure to warrior stress is unknown. Therefore, the present experiment evaluated the relationship between caffeine, stress, and symptoms of behavioral health disorders. To replicate the stress of combat (e.g., sensory stimulation and threat of death), this experiment used a warrior stress paradigm that has been used in previous rat experiments (4; 100; 104). When used in combination with sleep disruption techniques, it resembles acute and chronic stressors faced by deployed service members. This experiment examined three specific aims.

#### SPECIFIC AIM 1:

To examine the effects of caffeine on anxiety-like and depressive-like behaviors.

- ***Hypothesis 1a:*** Animals exposed to caffeine will demonstrate significantly greater anxiety-like behaviors on the open field activity (OFA) than animals not exposed to caffeine.
- ***Hypothesis 1b:*** Animals exposed to caffeine will demonstrate significantly less depressive-like behaviors on the OFA than animals not exposed to caffeine.

#### SPECIFIC AIM 2:

To examine the effects of stress on anxiety-like and depressive-like behaviors.

- ***Hypothesis 2a:*** Animals exposed to stress will demonstrate significantly greater anxiety-like behaviors on the OFA than animals not exposed to stress.

- **Hypothesis 2b:** Animals exposed to stress will demonstrate significantly greater depressive-like behaviors on the OFA than animals not exposed to stress.

### **SPECIFIC AIM 3:**

To examine the effects of caffeine on the relationship between stress and anxiety-like and depressive-like behaviors.

- **Hypothesis 3a:** Animals exposed to combined stress and caffeine will demonstrate significantly greater anxiety-like behaviors on the OFA than animals not exposed to stress.
- **Hypothesis 3b:** Animals exposed to combined stress and caffeine will demonstrate significantly greater depressive-like behaviors on the OFA than animals not exposed to stress.

**Rationale:** Deployed service members typically use caffeine in excess of recommended dosages to counter the adverse effects of a combat environment. The anxiogenic properties in caffeine heighten the senses, promoting increased awareness, alertness, and concentration among battle-fatigued service members. While caffeine has been reported to improve performance when consumed in doses of 250 mg or less, when taken in excess of 250 mg, caffeine results in symptoms of anxiety such as restlessness, nervousness, muscle twitching, psychomotor agitation, and tachycardia (3; 85; 86). Further, caffeine is associated with an increase in anxiety and vigilance for social threats (85; 86), which may increase service members' risk of developing anxiety and trauma-related disorders. Some studies indicate that caffeine may protect against depression (51; 60; 98), while others report that caffeine is associated with depression and anxiety disorders (27; 31; 76; 77; 84; 86; 92). However, these studies were: 1) not true

experiments and, 2) did not examine the interaction between stress, sleep disruption, and caffeine use on indices of behavioral health. The present experiment addressed these research gaps using an animal model.

## CHAPTER 3: Methods

To address the aforementioned hypotheses, this experiment employed a 2 (no caffeine, caffeine) x 2 (no stress, stress) with repeated measures (time: baseline, after 7 days stress, after 14 days of stress) factorial design. The four animal groups were: 1) no caffeine, no stress; 2) no caffeine, stress; 3) caffeine, no stress; and 4) caffeine, stress. Subjects were examined at three time points (i.e., baseline, after 7 days of stress, and after 14 days of stress; see Figure 1 for Experimental Timeline). There were eight subjects per condition. The number of subjects per condition was determined based on *a priori* power analysis and a long history of similar experiments conducted in the Grunberg Laboratory evaluating a variety of stressors and other similar predictor and outcome variables (4; 100; 104). This section provides an explanation of the subjects, housing conditions, power estimates, independent and dependent variables, experimental timeline, and data analysis.

### SUBJECTS AND HOUSING CONDITIONS

Subjects included 32 male Sprague-Dawley rats from Charles River Laboratories (Wilmington, Massachusetts) separated into four groups of 8 rats each. *A priori* power analysis using G\*Power indicated 6-8 rats per group would be sufficient to detect a large effect size with 80% power ( $\beta=.2$ ). Sprague-Dawley albino rats are known for their docile disposition and are widely used in animal research (34; 35; 69). Sprague-Dawley rats also are commonly used in caffeine research (48; 55). The animals were approximately 50-55 days old at the start of the study to closely approximate the age of military service members. According to the Department of Defense Office of the Deputy

Assistant Secretary of Defense (19), 43.1% of active duty service members and nearly 50% of active duty enlisted members were 25 years or less (53). The average age across all service components was 28.6 years old and 85.1% of the total active duty population was male (53). Further, Lieberman et al. (46) observed males consumed approximately 129 mg more per day of caffeine than females. In male and female Sprague-Dawley rats, adolescence is estimated between 20 and 60 days, with full adulthood beginning around day 60 (22). Based on the strain of rats used in similar caffeine research (48; 55) and current military demographic data (19), late adolescent/early adult Sprague-Dawley rats were considered the most appropriate strain for this experiment.

Animal care and housing was in accordance with policies outlined by the NIH Guide for Care and Use of Laboratory Animals (58). Animals were single housed in approved polycarbonate shoebox cages (42.5 x 20.5 x 20 cm) lined with hardwood chip bedding (Pine-Dri). Cages were maintained and cleaned twice weekly by the husbandry staff in Laboratory Animal Medicine (LAM) to ensure the rats did not experience additional stress because of poor housing conditions (68). The rooms were maintained at 23°C at 50% humidity on a 12 hour reverse light cycle to ensure measurement of nocturnal behaviors during the rats' active period (68). Animals had continuous access to food. Animals in the no caffeine condition had continuous access to water. Animals in the caffeine condition had continuous access to caffeinated-sucrose water, and for 2 hours each day, they were given access to a second home cage water bottle with pure tap water. Caffeine exposure via drinking water is explained in more detail below.

The USUHS Institutional Animal Care and Use Committee approved the protocol that governed this experiment (MPS-14-898; Behavioral investigations of nicotine and



caffeine in rats; see Appendix C). Experimenters also complied with guidelines set forth by the Guide for the Care and Use of Laboratory Animals (58).

## **INDEPENDENT VARIABLES**

There were three independent variables in this study: drug (no caffeine, caffeine), warrior stress (no stress, stress), and time (baseline, 7 days, and 14 days). All manipulation methods described in this section are similar to those conducted by Baisley (4) in the Grunberg Laboratory, which used this same animal model to examine the effects of repeated acute and chronic caffeine consumption on information processing in male rats.

### **Drug**

Animals in the drug condition were exposed to caffeine acutely via oral syringe and chronically via their home cage drinking water. These methods were selected based on previous research, findings from pilot studies (discussed in the next section), efforts to maximize face validity, and to ensure the animals did not experience undue stress from more invasive techniques such as intraperitoneal (IP) injection.

Chronic caffeine exposure (24 hours) occurred via the animals' home cage bottles (1 g/L in a 7% sucrose-water solution) and acute caffeine exposure (60 mg/kg in a 7% sucrose-water solution) was via oral syringe. These methods replicate service members' regular access to caffeine in the form of food and drink (e.g., coffee) and their access to pre-mission caffeine in the form of energy drinks. Animals in the non-caffeine condition were given 24-hour access to tap water.

### ***Pilot studies***

Dose levels and administration routes were selected based on findings from the animal literature and the outcomes from two pilot studies (4). The first pilot study examined two techniques to administer caffeine: 1) oral self-administration and, 2) caffeinated Jell-O. The pilot study used a 2 (male, female) x 2 (no caffeine, caffeine) x 2 (drinking water, Jell-O) factorial design with three animals per cell. For oral self-administration, animals in the caffeine condition received a caffeine-sucrose solution (1g/L of caffeine dissolved into a 5% sucrose solution), which was provided in their home cage bottles. Animals in the non-caffeinated condition received a 5% sucrose solution in place of tap water. Liquid consumption was measured every two to three days by weighing each water bottle and recording the start and end weights. Findings indicated that animals consuming pure sucrose water drank a considerable amount of sucrose solution. The water bottles of animals exposed to the 5% sucrose solution were often found near empty; consequently, these animals were consuming higher rates of sucrose compared to the animals exposed to the caffeine-sucrose solution. Given these findings, the present experiment exposed control animals to pure tap water only.

To replicate human caffeine exposure methods (e.g., drink and food) and to decrease stress on the animals from more invasive administration techniques (e.g., IP and gavage), Jell-O was considered as a potential administration route for the acute dose. This method is not widely used with caffeine; however, a previous study using Jell-O to administer buprenorphine concluded it was a viable drug exposure method (45). To assess the appropriateness of this method, animals were exposed to 2 mL of either caffeinated or non-caffeinated cherry Jell-O. Jell-O was presented to the animals in their

home cages. Results indicated the animals were not receptive to this method of caffeine administration and the animals either attacked or avoided the Jell-O boat. Ultimately, Jell-O was found to be a non-viable administration method for this experiment.

The second pilot was comprised of 12 rats and examined the use of syringe feeding via a feeding tube. Replicating the methods used by Schleimer et al. (80) which used syringes to administer haloperidol and diazepam, this experiment trained animals to consume 2 mL of sucrose solution via a feeding needle. Animal training involved gradually exposing the animals to a caffeine-sucrose solution. On the first day animals were exposed to a few drops of the solution and, over time, exposure gradually increased to 2 mL of the caffeine-sucrose solution. Administration involved placing the end of the feeding needle shallowly in the animal's mouth (not used as a gavage) and dispensing the liquid solution slowly. Based on previous caffeine research, pilot animals were exposed to moderate and high levels of caffeine (40 mg/kg and 80 mg/kg in 5% sucrose solution) to assess palatability of the caffeine/sucrose solution. Although all animals learned to consume the full 2 mL, animals receiving the lower dose appeared to consume the solution faster than animals receiving the 80 mg caffeine-sucrose solution. It is possible that the 80 mg caffeine-sucrose solution had a bitter and unpleasant taste. Based on these observations, 60 mg/kg of caffeine in a 7% sucrose solution was used for the present experiment. The overall findings from this pilot study indicated that animals were receptive to feeding via a syringe after a 3 to 4 day training period. Therefore, this method was used in the full experiment (discussed below).

Overall findings from the pilot studies suggested that: a) pure sucrose solution was consumed at a more rapid rate than the caffeine-sucrose solution and tap water; b)

Jell-O was not a feasible route for caffeine administration, c) caffeine exposure via a feeding needle was a viable administration method. Based on these findings, the present experiment used oral self-administration and syringe (parental) administration as the primary caffeine delivery methods to model the two ways that service members use caffeine.

### ***Oral self-administration, present experiment***

In the present experiment, animals had continuous access to caffeinated-sucrose via their home cage drinking water. Animals were exposed to caffeinated water 24-hours a day for the duration of the experiment, approximately 26 days. For 2 hours a day, animals in the caffeine condition were offered a choice between caffeinated-sucrose and pure tap water. The two-bottle choice was implemented to ensure appropriate hydration of the animals and to increase face validity. Caffeine-sucrose solution and tap water consumption rates were measured and recorded throughout the study. All liquids were refreshed every two to three days to avoid decomposition of the caffeinated-sucrose solution.

The caffeinated-sucrose solution in the home cage bottles was concentrated at 1 gram per liter (1 g/L) of caffeine and 7% sucrose. Sucrose was included as part of the caffeine solution to increase palatability and face validity. Caffeinated beverages such as energy drinks are typically high in sugar content. Caffeine concentration was considered a moderate to high dose based on prior caffeine studies (55; 59; 65; 74). Control animals received tap water based on results from the pilot studies and its use in other caffeine research (74). During the pilot studies, animals in the no-caffeine condition drank considerably more pure sucrose water than animals exposed to caffeinated-sucrose water.

To ensure non-caffeinated animals did not consume more sugar than the caffeinated animals, they were provided with an additional bottle of pure tap water for 2 hours a day. Additionally, regular and chronic consumers of caffeine may naturally consume more sugar than those consumers who abstain from caffeine.

### ***Syringe (parental) administration, present experiment***

Caffeine research indicates that caffeine consumption can increase endurance and improve performance (10; 28; 46). Deployed service members often consume an acute dose of caffeine prior to high-risk missions as a means to counter the effects of chronic sleep disruption and stress (10; 12; 49). To mirror the caffeine use of service members on deployment, animals in the present experiment were given an acute dose of caffeine prior to stress. Animals were exposed to the acute caffeine dose, concentrated at 60 mg/kg in 2 mL of 7% sucrose solution, via a syringe administration. Control animals received 2 mL of 7% sucrose solution. Syringes were fitted with a metal feeding needle (Figure 4), which was placed shallowly inside the animal's mouth.

### ***Preparation***

Animals were exposed to the caffeine sucrose solution via oral self-administration or syringe administration. Pure anhydrous caffeine (99.5% by HPLC) was purchased from Sigma-Aldrich Co., LLC and caffeine solutions were prepared using a Corning P-220 stirrer/hotplate (Corning, Inc., Corning, New York). The caffeine solution was created by first dissolving 7% sucrose solution into tap water. Second, caffeine was weighed, measured, and added to the solution to form a 1 g/L concentrated mixture. The final solution was poured into clean home cage water bottles and cooled to room temperature. Fresh caffeine sucrose solutions were produced twice weekly. Feeding

syringes also were prepared twice weekly and maintained in a refrigerator. The amount of caffeine for the 60 mg/kg sucrose solution was based on the average weight of the animals in the caffeine condition. Animals were weighed twice weekly, immediately prior to caffeine preparation. Animals in the caffeine condition were consistently within  $\pm 15\%$  of the average; therefore, all animals received the same solution.

### ***Syringe feeding acclimation***

After baseline data were collected (described below), animals underwent a three-day syringe-training period with a 5% sucrose solution. Sucrose solution was used during training so the animals would associate parental feeding with the pleasant taste of sucrose water and not the aversive taste of caffeine. The syringe was presented to the animals by placing the end of the feeding needle shallowly inside each animal's mouth. On the first day, animals were exposed to a few droplets of solution. On the second day, animals were exposed to 1 mL of the 7% sucrose solution. On the third and final day, animals were exposed to 2 mL of the sucrose solution.

### **Warrior stress paradigm (WSP)**

The Grunberg Laboratory created the Warrior Stress Paradigm (WSP) to model stressors encountered in combat settings. Several studies have found the WSP to be a valid model for replicating the biological and behavioral responses commonly experienced by service members exposed to acute and chronic stress (6; 34; 104). The WSP is considered a mild to moderate, unpredictable stress paradigm. The WSP exposes animals to predator scent and a series of unpredictable sensory stressors (Figure 2) that are common components in animal stress paradigms (30). The WSP is administered for 20 min/day.

Predator cues include exposure to live predators (e.g., a cat), predatory cues (e.g., cat or fox urine), or a combination of predator cues and sensory stressors such as coin shaking in a metal can, flashing lights, and cage rattling in a single exposure (1; 2; 20). To elicit fear from predator cues, animals are exposed to a cotton ball saturated with fox urine for various lengths of time (14). The literature indicates that predator scent (i.e., cat or fox urine) is an appropriate and realistic stressor to evaluate natural emotional and behavioral responses in rats (1; 2; 20). Additional unpredictable sensory stimuli included coin shaking (i.e., from a person shaking a can filled with coins), flashing lights (i.e., from a person repeatedly turning the lights on and off), and cage rattling (i.e., from a person shaking the animal's cage) for various lengths of time (4; 54; 100; 104) (Figures 2 and 3).

### ***Warrior stress and sleep disruption (SD)***

In the latter half of the experiment, animals in the stress condition were exposed to the WSP and sleep was disrupted. The sleep disruption component has been validated in previous Grunberg Laboratory studies and when incorporated into the WSP, it elevates the intensity of the stress model (63; 70). The SD manipulation consisted of exposure to sounds of environmental stressors and sleep disrupting stimuli. During this phase, animals were transported from their home cage rooms into a smaller unfamiliar room during their normal sleep period. While in the sleep disruption room, animals were exposed to sleep disrupting environmental sounds such as honking horns, crashing glass, bells, and voices. These noises were recorded on a CD and delivered using a standard CD-player set at a volume that emitted high and low frequency noises between 65 to 80 decibels. Sleep disrupting sounds played on an hourly loop, 9 hours a day (2200 to

0700), for seven consecutive days. Total duration of sound exposure ranged from 6 to 60 seconds, and did not exceed 6 minutes at any time during this phase (63; 70).

### **Time**

Time was examined as a between-subjects variable. Three separate time points were investigated to determine whether caffeine resulted in higher anxiety-like behaviors in animals exposed to the WSP. Animals were examined at baseline, 7 days, and 14 days.

### **DEPENDENT VARIABLES**

The dependent variables were anxiety-like and depressive-like behaviors as measured by the open field activity (OFA; Figure 5). Assessment of general motor functioning involved calculation of horizontal activity. Assessment of anxiety-like behaviors on the OFA involved examination of locomotor activity and the ratio of time spent in the center of the field compared to total movement time. Assessment of depressive-like behaviors on the OFA involved calculation of vertical activity.

### **Open field activity (OFA)**

The open field activity (OFA) is commonly regarded as a valid and reliable measure to assess general motor function as well as anxiety-like and depressive-like behaviors among rodents (5; 23; 61; 69). Three parameters frequently used to assess motor functioning, anxiety-like behaviors, and depressive-like behaviors are horizontal activity (HA), center time (CT), and vertical activity (VA), respectively.

The OFA is a procedure that measures rodent activity by counting the number of infrared beams the animal breaks in various quadrants of the chamber. Rodents are



placed in a clear box for 60 min, during which time their vertical and horizontal movements are tracked and recorded using the Omnitech/Accuscan Electronics Digiscan infrared photocell system (Figure 5).

Examination of HA, or the total number of beam breaks in the horizontal plane, is used to assess general health and motor functioning. To assess anxiety-like behaviors, the ratio of time spent in the center of the field is compared to total movement time. Animals generally prefer moving along the periphery of the apparatus, and avoid extended periods in the center of the box (69). Higher rates of time spent in the center field increases the animals' vulnerability to predator attack. Therefore, high rates of CT are indicative of low anxiety-like behaviors (69). To assess depressive-like behaviors, VA is calculated by counting the total number of beam breaks in the top quadrant of the OFA. These procedures are based on the Learned Helplessness Model, which posits that healthy animals should attempt escape (78). Rearing behaviors are considered escape behaviors. As such, lower rates of rearing activities (i.e., fewer vertical beam breaks) are indicative of depressive-like behaviors.

In their review of OFA as a measure of pharmacological effects, Prut and Belzung (69) confirmed the construct validity of OFA. Each session, animals were placed in the OFA chamber for 1 hour. System software tracked the following for each animal: total movement time, total vertical activity, total horizontal activity, and time spent in the center of the chamber. To decrease the likelihood of habituation or conditioned place preferences, animals were placed in a different OFA chamber during each iteration of testing.

## **PROCEDURES**

The study procedures closely mirrors other studies by the Grunberg Laboratory (4; 54; 100; 104) and is closely associated with a study by Baisley (4), which used the same animals to examine the combined effects of stress, caffeine, and sleep disruption on measures of information processing in rats. See Figure 1 for a visual representation of the experimental timeline.

### **Acclimation phase**

Animals underwent both a gentling and habituation period (3 days), during which time the animals were exposed to gentle human touch for 2 min each and then numbered for accountability and tracking purposes. Following gentling and numbering, each animal spent 1 hour acclimating to the open field activity (OFA; Figure 5).

### **Baseline phase**

After gentling, numbering, and acclimation procedures were complete, researchers collected baseline behavioral information (i.e., the animal's natural horizontal, vertical, and exploratory locomotor activity) on each measure. Each animal was placed into an electronic physical activity-monitoring chamber of the Omnitech/Accuscan Electronics Digiscan infrared photocell system for 1 hour to measure locomotor activity in the OFA. Researchers cleaned the OFA with a 70% ethanol treatment after each test to eliminate the odor of the previous animal. Collection of baseline data occurred during the active (dark) phase of the light cycle.

### **Syringe training phase**

After baseline data were collected, animals underwent three days of syringe training (described above). By the conclusion of training, animals were exposed to the same amount of solution (2 mL) as their acute dose.

### **Drug administration phase**

Caffeinated water was presented to the animals in the caffeine condition immediately following baseline data collection. Animals in the caffeine condition were exposed to the caffeine-sucrose solution in their home cage water bottles for 28 days. To prevent the possibility of dehydration and to increase face validity, an additional water bottle filled with tap water was placed in their home cages for 2 hours a day. Animals in the control condition were allowed continuous access to tap water via their home cage water bottles.

### **Warrior stress phase**

Animals in the stress condition were exposed to the warrior stress paradigm (WSP) for two, 7-day, stress iterations (14 days total). Each 7-day period was followed by three days of behavioral testing. During the first iteration, animals were transported from their home cages into another room where they were placed in smaller mice cages (19 x 18 x 12 cm). On the first day, animals were exposed to a cotton ball soaked in fox urine for 20 minutes. On subsequent stress days, animals were exposed to the fox urine for 10 minutes and then unpredictable environmental stressors for 10 minutes (Figure 2). The total stress time was approximately 20 minutes. During the second iteration, animals were exposed to the WSP and sleep disruption stressors (see above).

## **CHAPTER 4: Data Analytic Strategy**

### **OPEN FIELD ACTIVITY (OFA)**

Repeated-measures analyses of covariance (rANCOVAs) were conducted to assess within-group and between-group differences in the open field activity (OFA) on the independent variables of caffeine and stress (Tables 17, 20, and 22). rANCOVAs were used for each dependent variable: 1) general locomotor activity as measured by horizontal activity (HA), 2) anxiety-like behaviors as measured by the ratio of center time (CT), and 3) depressive-like behaviors as measured by vertical activity (VA). Baseline scores were used as covariates to account for pre-existing individual differences among the animals. An examination of the data from all 32 subjects revealed two animals were outliers, one from the caffeine-stress and one from the caffeine-only condition. These animals had extreme scores (one animal was  $>3$  SDs and one animal approached 3 SDs from the group means) at time two on HA, and were subsequently removed from the final analysis.

The OFA measured locomotor activity of the animals using 16 infrared beams along the X and Y grid axes. The animals' horizontal and ambulatory activity was measured by calculating their overall number of beam breaks. Horizontal activity ranged from 0 to 12,000 beam breaks with higher numbers representing more locomotion. Anxiety-like behaviors were measured by calculating the ratio of center time (i.e., the amount of time the animal remained in the center of the field) compared to total movement time. At time one, the ratio ranged from .03 to .28. At time two, the ratio ranged from .02 to .39. Higher numbers indicated greater time spent in the center of the field and lower levels of anxiety. The animals' vertical activity (based on number of

beam breaks) was also measured. Vertical activity ranged from 0 to 2,000 with higher numbers representing more escape-like behaviors and less depression. Animal movements were tracked and recorded using the Omnitech/Accuscan Electronics Digiscan infrared photocell system.

#### **WATER AND CHRONIC CAFFEINE CONSUMPTION**

Home cage water bottles were refreshed and consumption data were collected every three to four days. Data were recorded at six time points over the course of the experiment. To estimate liquid consumption, univariate analyses of variance (ANOVA) were run to identify between-group differences at each time point (Tables 2 to 8 and Figure 6). Caffeine consumption estimates were calculated after accounting for animals' body weight, average natural leakage from the water bottles, and the amount of caffeinated-sucrose solution consumed (Tables 9 to 15, and Figure 7).

## **CHAPTER 5: Results**

### **DAILY LIQUID CONSUMPTION**

Home cage water bottles were weighed every 3 or 4 days. Descriptive data of daily liquid consumption are presented in Table 2. Figure 6 shows daily liquid consumption over time by group. ANOVAs at each of the six time points revealed a significant main effect of caffeine at time one ( $F[1, 26]=6.65, p=.02, \eta_p^2=.20$ ) and time 3 ( $F[1, 26]=4.52, p=.04, \eta_p^2=.15$ ) with the caffeine group consuming significantly more liquid (i.e., tap water and caffeine-sucrose solution) than the non-caffeine group (Tables 3 and 5). There were also significant caffeine by stress interactions at times five ( $F[1, 26]=6.42, p=.02, \eta_p^2=.20$ ) and six ( $F[1, 26]=5, p=.03, \eta_p^2=.16$ ) indicating that the stressed animals in the caffeine group consumed less liquid than controls and non-stressed animals in the caffeine group consumed more liquid than controls (Tables 7 and 8).

### **CAFFEINE CONSUMPTION**

Descriptives of caffeine consumption (self-administered) are presented in Table 9. Caffeine consumption was compared between the stressed and non-stressed animals for each of the six time points (Tables 10 to 16). Overall, there were no significant differences between the stressed and non-stressed animals in total amount of caffeine consumed across the six time points (Figure 7). Caffeinated animals consumed between 3-30 mg/kg of caffeine a day (excluding the acute dose). Based on the delivery method of the acute dose (i.e., syringe administration), it is estimated that animals in the stress condition were exposed to approximately 1 to 2 mL of the 60 mg/kg caffeine-sucrose solution. During the experiment, researchers observed that some of the caffeine-sucrose

solution leaked out of the animals' mouths during the syringe administration; therefore, precise consumption rates of the acute dose cannot be provided.

### **HORIZONTAL ACTIVITY (HA)**

The overall rANCOVA (Table 17 and Figure 8) indicated there was no significant main effect of time ( $F[1, 25]=2.19, p=.15, \eta_p^2=.08$ ), no significant main effect of caffeine ( $F[1, 25]=2.91, p=.10, \eta_p^2=.10$ ), and no significant main effect of stress ( $F[1, 25]=.45, p=.51, \eta_p^2=.02$ ). There also was no significant caffeine x stress interaction ( $F[1, 25]=2.25, p=.15, \eta_p^2=.08$ ).

Although there were no significant main effects, further examination of the data indicated significant differences at time two. An ANCOVA, holding baseline HA scores as covariates, revealed a significant difference ( $F[1, 25]=33.05, p<.01, \eta_p^2=.57$ ) in horizontal activity at time two between caffeinated and non-caffeinated animals (Table 18). Animals in the caffeine group ( $M=6451.93, SD=1524.12$ ) showed significantly less general locomotion than animals in the no-caffeine condition ( $M=9182.63, SD=1873.76$ ; Table 16). The ANCOVA did not show a significant effect of stress.

### **Summary of Horizontal Activity**

Overall, there were no significant main effects and no interaction; however, additional analysis at time two showed the caffeine group had significantly less locomotion than the non-caffeinated control group. It is possible that, over time, acute and chronic caffeine exposure results in decreased general movement.

### **CENTER TIME (CT/MT)**

The overall rANCOVA (Table 20 and Figure 9) indicated there was no significant main effect of time ( $F[1, 25] = .01, p = .94, \eta_p^2 = 0$ ), no significant effect of caffeine ( $F[1, 25] = 0, p = .95, \eta_p^2 = 0$ ), and no significant effect of stress ( $F[1, 25] = .1, p = .76, \eta_p^2 = .004$ ). Also, there was no significant caffeine x stress interaction ( $F[1, 25] = .05, p = .82, \eta_p^2 = .002$ ).

### **Summary of Center Time**

Analysis of center time indicated no significant within or between-group differences. All animals, regardless of group, spent a similar amount of time in the center of the open field compared to their total movement time.

### **VERTICAL ACTIVITY (VA)**

The overall rANCOVA (Table 22 and Figure 10) indicated there was no significant main effect of time ( $F[1, 25] = 1.80, p = .19, \eta_p^2 = .07$ ). There was a significant effect of caffeine ( $F[1, 25] = 6.71, p = .02, \eta_p^2 = .21$ ) with the control group ( $M = 1629.63, SE = 118.38$ ) evidencing significantly more vertical activity than the caffeine group ( $M = 1180.60, SE = 126.55$ ) (Table 21). There was no significant effect of stress ( $F[1, 25] = .38, p = .55, \eta_p^2 = .02$ ). There also was no significant caffeine x stress interaction ( $F[1, 25] = 2.12, p = .16, \eta_p^2 = .08$ ).

Separate ANCOVAs (with baseline vertical activity scores as covariates) at each time point indicated no significant between-group differences at time one (Table 23). However, there was a significant effect of caffeine at time two ( $F[1, 25] = 10.63, p < .01$ ,



$\eta_p^2=.30$ ), where the caffeine group ( $M=968.00$ ,  $SD=275.38$ ) evidenced significantly less vertical activity than the non-caffeine control group ( $M=1400.69$ ,  $SD=449.82$ ; Table 21).

### **Summary of Vertical Activity**

Analysis of vertical activity revealed a significant effect of caffeine, with the caffeinated animals exhibiting less vertical activity than the non-caffeinated animals. Further examination of the data at each time point revealed a significant difference at time point two with the caffeine animals exhibiting significantly less vertical activity than non-caffeine animals. These data indicate that caffeine reduced escape activity and suggests that, over time, caffeine use may lead to an increase in depressive-like behaviors.

## CHAPTER 6: Evaluation of Hypotheses

### SPECIFIC AIM 1:

To examine the effects of caffeine on anxiety-like and depressive-like behaviors.

- ***Hypothesis 1a:*** Animals exposed to caffeine will demonstrate significantly greater anxiety-like behaviors on the OFA than animals not exposed to caffeine. The hypothesis that caffeine would increase anxiety-like behaviors was **not supported**. The results indicated non-significant effects of experimental manipulations as measured by the ratio of center time to movement time on the OFA.
- ***Hypothesis 1b:*** Animals exposed to caffeine will demonstrate significantly less depressive-like behaviors on the OFA than animals not exposed to caffeine. The hypothesis that caffeine would decrease depressive like behaviors on the OFA was **not supported**. Instead, the results indicated **significant effects of caffeine exposure to increase depressive-like behaviors** as measured by vertical activity on the OFA. Separate analyses at each time point indicated a significant difference at time two, where the caffeine group evidenced significantly less vertical activity than the non-caffeine control group. These data indicate that caffeine reduced escape behaviors.

### SPECIFIC AIM 2:

To examine the effects of stress on anxiety-like and depressive-like behaviors.

- ***Hypothesis 2a:*** Animals exposed to stress will demonstrate significantly greater anxiety-like behaviors on the OFA than animals not exposed to stress. The hypothesis that stress would increase anxiety-like behaviors was **not supported**.

The results indicated non-significant effects of experimental manipulations as measured by the OFA.

- ***Hypothesis 2b:*** Animals exposed to stress will demonstrate significantly greater depressive-like behaviors on the OFA than animals not exposed to stress. The hypothesis that stress would increase depressive-like behaviors was **not supported**. The results indicated non-significant effects of experimental manipulations as measured by the OFA.

### **SPECIFIC AIM 3:**

To examine the effects of caffeine on the relationship between stress and anxiety-like and depressive-like behaviors.

- ***Hypothesis 3a:*** Animals exposed to stress and caffeine will demonstrate significantly greater anxiety-like behaviors on the OFA than animals not exposed to stress and caffeine. The hypothesis that combined caffeine and stress would increase anxiety-like behaviors was **not supported**. The results indicated non-significant effects of experimental manipulations on measures of anxiety.
- ***Hypothesis 3b:*** Animals exposed to stress and caffeine will demonstrate significantly greater depressive-like behaviors on the OFA than animals not exposed to stress and caffeine. The hypothesis that combined stress and caffeine would increase depressive-like behaviors was **not supported**. The results indicated non-significant effects of experimental manipulations as measured by the OFA.

## CHAPTER 7: Discussion

Caffeine is commonly consumed by military service members with upwards of 45% regularly ingesting caffeine in amounts exceeding 300 mg per day (12). While an abundance of literature highlights the positive effects of moderate caffeine consumption, studies also suggest that doses exceeding 300 mg/day can result in symptoms of anxiety and heightened arousal (85; 86). Research examining effects of caffeine on depression has produced mixed results. Some research suggests caffeine may increase the risk of depression (27; 31; 76; 84; 92) and other research suggests it may serve in a protective role (51; 60).

Using an animal model, the present experiment examined effects caffeine exposure and stress on indices of behavioral health. The independent variables manipulated in this experiment included: caffeine (no caffeine, caffeine) and stress (no stress, warrior stress paradigm [WSP] + sleep disruption). Caffeine was administered in two forms for chronic and acute consumption. These exposure methods were selected to model the manner in which service members consume caffeine. While deployed, some service members consume caffeine regularly throughout the day (e.g., coffee, coke, and food) and consume a pre-mission energy drink prior to leaving their forward operating base or command outpost. Therefore, animals were exposed to caffeine daily via their home cage drinking water and pre-stress via a feeding syringe (Figure 4). To model stressors encountered in a deployed environment, this study employed a nested stress paradigm, which included 7 days of mild warrior stress followed by 7 days of warrior stress and sleep disruption. The WSP is a stress paradigm that closely mirrors environmental stressors in the deployed environment (Figure 2). During the first 7 days

of stress, animals in the stress condition were exposed to unpredictable sensory stressors such as fox urine (predator scent), flashing lights, whistle blasts, enclosed spaces, cage shaking, and coin rattling (Figure 3). During the last 7 days of stress, animals in the stress condition also were exposed to 9 hours of high and low frequency noises designed to disrupt their sleep cycle.

To assess the impact of stress and caffeine on proxies of behavioral health, the following dependent variables were assessed: anxiety-like behaviors and depressive-like behaviors. It was hypothesized that animals exposed to caffeine would demonstrate greater anxiety-like and less depressive-like behaviors on the open field activity (OFA; Figure 5); animals exposed to stress would evidence greater anxiety-like and depressive-like behaviors on the OFA; and animals exposed to both stress and caffeine would demonstrate greater anxiety-like and depressive-like behaviors on the OFA.

The OFA was used to assess behaviors indicative of anxiety and depression. Specifically, the ratio of center time compared to movement time was examined as a proxy of anxiety. Animals prefer dark spaces, corners, and moving along the periphery of the apparatus (69). Animals generally avoid extended periods in the center of the box because it increases their vulnerability to predator attack. Therefore, animals with greater time spent in the center of the field (as compared to overall movement time) are considered less anxious than those with less center time (69). Depressive-like behaviors were measured by calculating the total number of vertical beam breaks in the upper quadrant of the OFA. Reduced escape behaviors are considered an indicator of depression (78). Therefore, animals with less vertical activity (VA) are considered more depressed than animals with higher rates of VA. The remainder of the section will

examine study findings based on the outcome variables measured, review limitations, and propose future directions.

## **OUTCOME VARIABLES**

### **Water and caffeine consumption**

There were significant between-group differences in total liquid consumption at times one and three with the caffeine group consuming more liquid than the non-caffeine group (Tables 3 and 5). There were significant caffeine by stress interactions at times five and six (Tables 7 and 8) indicating that the stressed animals in the caffeine group consumed less liquid than controls and non-stressed animals in the caffeine group consumed more liquid than controls. There were no significant between-group differences in total caffeine consumed, indicating that animals in the caffeine-stress and caffeine-only groups consumed roughly the same amount of caffeine. Overall, animals in the caffeine condition consumed between 3 and 30 mg of caffeine a day (not including caffeine from the acute dose). Animals exposed to the acute dose consumed on average 1 mL of the 60 mg/kg caffeine-sucrose solution. Animals metabolize oral caffeine approximately 10 times faster than humans (97). Therefore, a service member weighing 190 pounds (86.4 kg) who consumes between 350 mg to 1000 mg of caffeine a day is consuming approximately 4 to 12 mg/kg of caffeine a day. The animals in this study consumed 0.3 to 3 mg/kg/day, which was lower than the high amounts consumed by some service members (i.e., 4 to 12 mg/kg/day).

### **Locomotion: Horizontal activity (HA)**

Overall, there were no significant main effects of caffeine or stress on general locomotion as measured by HA. Analysis at time two showed caffeinated animals had

less exploratory behavior than controls, which may be an indicator of habituation.

However, the findings from the present study differ from other research in the field.

Rhoads et al. (74) compared acute and adaptive motor responses in adult and adolescent

Long-Evans rats. They found that adult rats evidenced a high initial burst of activity and

consistent movement after caffeine exposure. Adolescent rats evidenced increased

movement at moderate dosages of caffeine (e.g., 20-30 mg/kg). Noschang et al. (59)

examined the effects of caffeine and chronic stress on anxiety-like behaviors in male and

female Wistar rats. Their data indicated that animals exposed to caffeine at 1 g/L showed

no changes in motor activity, which they attributed to tolerance. Overall, these discrepant

findings may be related to differences in the animal strains.

#### **Anxiety: Center time/movement time (CT/MT)**

The animals' levels of anxiety were measured by calculating the ratio of center time (i.e., the amount of time the animal remained in the center of the field) to total movement time. The animals' preference for enclosed spaces is similar to a deployed service member's preference for environments that provide good cover and concealment. Long periods spent in open areas can increase a service member's risk of exposure to enemy fire. Similarly, if animals spend increased time in the center of the field, then they are more vulnerable to predator attack. Consequently, greater time spent in the center of the open field is indicative of lower anxiety. Analysis of center time indicated no significant within or between-group differences.

The animals did not show significantly different anxiety-like behaviors over time suggesting that caffeine did not exacerbate the effects of stress. These findings differ from Noschang et al. (59). Their data indicated male rats exposed to caffeine spent less

time in the center of the OFA, suggesting higher levels of anxiety-like behaviors. Of note, Noschang et al. (59) also found evidence of increased anxiety-like behaviors on the elevated plus maze, a validated measure to assess anxiety-like behaviors in rats. The differences between the present study's findings and Noschang et al. (59) findings may be due to large differences in the animals' consumption rates and strain of rat. Animals in the Noschang et al. (59) study were exposed exclusively to caffeinated-water (whereas the present study allowed the animals access to tap water for 2 hours a day). The Noschang et al. (59) animals self-administered considerably more caffeinated-water than animals in this study. Consequently, animals in their study may have consumed a higher caffeine dosage than animals in the present study.

#### **Depression: Vertical activity (VA)**

Escape-like behaviors (e.g., rearing) are common among healthy rats. Research based on the learned helplessness model indicates that low levels of movement and escape behaviors are indicative of depression (43; 54; 78; 100; 104). The animals' vertical activity was examined by counting the number of infrared beams breaks in the top quadrant of the OFA. Animals with fewer beam breaks demonstrated less escape behaviors, which is an indication of depression. Analysis of vertical activity indicated no significant effect of time and no significant effect of stress; however, there was a significant effect for caffeine. Significant group effects showed that the caffeine animals evidenced significantly less vertical activity than controls. Separate analyses at each time point indicated a significant between-group difference at time two with the caffeine group evidencing significantly less vertical activity than the no-caffeine control group.



Overall, these data suggest that the chronic use of caffeine may, over time, lead to an increase in depressive-like behaviors. These findings are different from findings by Pechlivanova et al. (61) and Pechlivanova et al. (62), which indicated caffeine dose-dependently reversed depressive-like behaviors in rats exposed to chronic stress. Both these studies examined the acute effects of caffeine, not the combined acute and chronic effects of caffeine as examined by the present experiment. Further, Pechlivanova et al. (62) administered caffeine via gavage at 8 mg/kg/day and Pechlivanova et al. (61) used 2, 20, 40 mg/kg delivered intraperitoneally, which was a considerably lower dosages than used in the present experiment. Despite their findings, Pechlivanova et al. (62) suggest that long-term caffeine exposure can create effects similar to those created by a chronic unpredictable stress model (e.g., low locomotion and low anxiogenic effects). Differences in caffeine administration and dosing likely explain the differential findings.

## **LIMITATIONS**

### **Animal model**

Although animal models are useful for examining human problems, they are not perfectly translatable. The present study attempted to model the human deployed experience; however, there are several limitations. In addition to problems with administration of the manipulations (discussed below), the current study only used one apparatus for measuring anxiety-like and depressive-like behaviors. Technical problems precluded the use of additional measures to assess outcome variables. Extant literature supports using multiple measures (71; 81); therefore, inclusion of measures such as the Elevated Plus Maze to assess anxiety, and the Forced Swim Test to assess depression, would be important in future experiments.

The present experiment only examined the effects of caffeine and stress on proxies of behavioral health in male rats. According to Department of Defense Office of the Deputy Assistant Secretary of Defense (19), men comprise 85.5% of the DoD active duty force. Because this is the first study to evaluate the combined effects of caffeine and warrior stress using a novel and face valid model of caffeine administration, the experiment used male sex rats. Female sex rats may produce different results providing a fuller explanation of these combined variables on behavioral health. Future studies should consider including female sex rats.

### **Caffeine**

The study was designed to maximize face and ecological validity. As such, researchers used drinking water and feeding syringes as the methods of caffeine exposure. Animals exposed to the acute caffeine dose only consumed approximately 1 mL of the 2 mL caffeine-sucrose solution. Also, animals in the caffeine condition self-administered less caffeine than animals in other similar studies (59). Consequently, caffeine levels may not have reached the target dose. It is likely that animals in the caffeine condition failed to consume enough caffeine to mirror the high doses consumed by service members. Future research should use a drug administration method that allows for greater control of dosing levels (e.g., gavage or intraperitoneal) (55; 61; 62; 72).

Another limitation involved metabolism and the timing of stress manipulations. The time between the acute dose and stress exposure varied for each animal and ranged from 30 minutes to 60+ minutes. Therefore, it is unclear to what degree the caffeine had been metabolized by the animals prior to stress exposure. Future studies should consider

counter-balancing the time of the acute dose administration to the individual animals based on experimental condition.

### **Warrior Stress Paradigm (WSP)**

The WSP was developed by the Grunberg Laboratory to mimic the stressors of combat deployment. The current paradigm consists of sensory stressors such as whistle blasts, exposure to fox urine, flashing lights, and cage shaking. However, it is unclear to what degree the paradigm is effective in creating an adequate stress response. Although the WSP has demonstrated effects in some Grunberg Laboratory research (4; 6; 34; 104), collection of physiological stress markers (e.g., corticosterone) would have allowed validation of the stress effects in the present experiment.

### **Sleep Disruption (SD)**

Animals in the sleep disruption (SD) group were moved from their usual living environment to another room in the facility. A variety of high and low frequency noises were played for 6 to 60 seconds on a 9-hour loop at decibels ranging from 65-80. There was no manipulation check of the SD paradigm, so it is unclear to what degree the animals' sleep was disrupted during this exposure. Further, since SD was implemented as part of the WSP during the latter half of the experiment, the effects of SD cannot be examined in isolation. A more informative approach should include three separate groups: 1) warrior stress only, 2) sleep disruption only, and 3) combined warrior stress and sleep disruption.

## **FUTURE DIRECTIONS**

### **Animal model**

Correcting limitations outlined in the previous section is important for future research. The present study used a common and widely accepted approach to examine anxiety-like and depressive-like behaviors in rats. However, extant literature supports using multiple measures to examine behavior (71; 81). Inclusion of measures such as the Elevated Plus Maze to assess anxiety-like behaviors and the Forced Swim Test to assess depressive-like behaviors will help elucidate findings. Finally, the inclusion of female rats would allow researchers to more fully appreciate potential gender differences when examining the effects of caffeine and stress on behavioral health.

### **Caffeine**

The present study attempted to maximize ecological and face validity by exposing animals to caffeine via routes commonly used by humans. However, because animals self-administered caffeine, researchers could not ensure they were exposed to the target dose of caffeine. Further, the preponderance of research has identified low, moderate, and high caffeine doses for IP, not feeding syringes. Therefore, future research should consider using more established methods of caffeine administration (e.g., IP) to ensure caffeinated animals are exposed to the desired dose. The collection of biological markers would help confirm caffeine levels in exposed animals. Finally, it is recommended that future research apply a counterbalanced approach to administer the acute dose to account for time differences between acute administration and warrior stress exposure.

## **Warrior stress and sleep disruption**

The Grunberg Laboratory created the WSP to replicate stressors encountered in a combat deployment. The WSP was administered during the first 7 days of stress and a SD component was added during the second 7 days of stress. Future studies should consider creating three stress groups: 1) WSP, 2) SD, and 3) a combined WSP+SD group to allow for a closer examination of the stressors. Additionally, collection of stress biomarkers (e.g., corticosterone) would allow researchers to determine the effectiveness of the WSP in producing biological stress responses in rats.

## **Implications**

Military service members, both deployed and non-deployed, face a high number of acute and chronic stressors which can have deleterious effects on behavioral health. Caffeine is a widely used performance-enhancing supplement, used to counter the negative effects of stress and fatigue. Findings from the present experiment indicate that chronic caffeine use at moderate doses may contribute to the development of behavioral health symptoms associated with depression in an animal model. Given the increasing rates of behavioral health problems across the military, commanders should consider advocating for longitudinal research that examines the overall functioning of service members exposed to high levels of caffeine consumption, stress, and sleep disruption. Commanders also can provide education to service members on both the positive and negative effects of caffeine, based on the scientific literature. Military leaders and policy writers should examine the accessibility of highly caffeinated beverages and consider whether the military's culture around high caffeine use should be changed. Finally,

clinicians should examine caffeine as a contributing factor in the development and maintenance of depression-related behavioral health problems.

## **CHAPTER 8: Summary**

The present study attempted to model caffeine consumption and stress for male service members operating in a combat environment. In an effort to maximize ecological and face validity, animals were exposed to acute and chronic caffeine via administration methods commonly used by humans. Instead of using more invasive routes such as intraperitoneal (IP), animals were exposed to caffeine via their drinking water and a feeding syringe. Similarly, a warrior stress paradigm (WSP) was employed to replicate stressors encountered in a combat environment. During the second phase of stress, sleep disruption (SD) was implemented as part of the WSP to replicate sleep conditions during deployment.

The experiment did not reveal any significant effects of caffeine or stress on indices of anxiety-like behaviors in rats. This finding may be the result of limitations with the WSP and/or problems with caffeine dosing levels. However, the study did reveal a significant effect of caffeine on indices of depression. These findings suggest that caffeine, over time, may lead to increased levels of depression in exposed animals.

## **CHAPTER 9: Conclusions**

There were no clear effects for caffeine or stress on anxiety-like behaviors in rats. As such, the hypotheses concerning anxiety-like behaviors were not supported. There were, however, effects of caffeine on markers of depression. Overall, caffeine had a deleterious effect on behaviors related to depression.



## APPENDIX A: Tables

Table 1: Study Design

Repeated-Measures Full Factorial Mixed Design (N=32)		
	No Caffeine	Caffeine
No Stress	8	8
Stress*	8	8
*Stress days 0-7 used the warrior stress paradigm; stress days 8-14 used the warrior stress paradigm combined with sleep disruption		

Table 2: Descriptives [Means (SDs)] of Total Liquid Consumption over Time (in mL)

	Days -6 to -1 (Time 1)	Days 0 to +2 (Time 2)	Days +3 to +6 (Time 3)	Days +7 to +9 (Time 4)	Days +10 to +13 (Time 5)	Days +14 to +17 (Time 6)
Control	38.23 (7.5)	45.07 (7.01)	35.05 (9.48)	42.40 (12.73)	36.72 (10.02)	39.17 (9.50)
Stress	41.27 (9.45)	49.06 (13.05)	36.42 (6.26)	43.55 (15.88)	47.48 (11.96)	50.61 (10.82)
Caffeine	49.94 (9.81)	55.67 (11.09)	45.15 (18.92)	46.83 (20.60)	50.42 (13.44)	53.26 (16.94)
Caffeine- Stress	45.28 (5.90)	53.97 (14.28)	44.50 (9.15)	37.73 (7.40)	41.20 (6.20)	46.01 (6.00)
No shading indicates half-caffeine concentration in drinking water (1/2 g/L) Lighter shading indicates WSP at time of data collection Darker shading indicates WSP+SD at time of data collection						

Table 3: ANOVA of Total Liquid Consumption (in mL) Time 1

Tests of Between-Subjects Effects TtlVol1 (Days -6 to -1)					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	3	2.76	0.06	0.24	0.60
Intercept	1	820.97	0.00	0.97	1.00
Caffeine	1	6.65	0.02	0.20	0.70
Stress	1	0.07	0.79	0.00	0.06
Caffeine*Stress	1	1.59	0.22	0.06	0.23
Error	26				
Total	30				
Corrected Total	29				

Table 4: ANOVA of Total Liquid Consumption (in mL) Time 2

Tests of Between-Subjects Effects TtlVol2 (Days 0 to 2)					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	3	1.29	0.30	0.13	0.30
Intercept	1	576.33	0.00	0.96	1.00
Caffeine	1	3.33	0.08	0.11	0.42
Stress	1	0.07	0.79	0.00	0.06
Caffeine*Stress	1	0.45	0.51	0.02	0.10
Error	26				
Total	30				
Corrected Total	29				

Table 5: ANOVA of Total Liquid Consumption (in mL) Time 3

Tests of Between-Subjects Effects TtlVol3 (Days 3 to 6)					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	3	1.53	0.23	0.15	0.36
Intercept	1	354.67	0.00	0.93	1.00
Caffeine	1	4.52	0.04	0.15	0.53
Stress	1	0.01	0.93	0.00	0.05
Caffeine*Stress	1	0.06	0.82	0.00	0.06
Error	26				
Total	30				
Corrected Total	29				

Table 6: ANOVA of Total Liquid Consumption (in mL) Time 4

Tests of Between-Subjects Effects TtlVol4 (Days 7 to 9)					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	3	0.45	0.72	0.05	0.13
Intercept	1	244.37	0.00	0.90	1.00
Caffeine	1	0.02	0.90	0.00	0.05
Stress	1	0.53	0.47	0.02	0.11
Caffeine*Stress	1	0.88	0.36	0.03	0.15
Error	26				
Total	30				
Corrected Total	29				

Table 7: ANOVA of Total Liquid Consumption (in mL) Time 5

Tests of Between-Subjects Effects TtlVol5 (Days 10 to 13)					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	3	2.48	0.08	0.22	0.55
Intercept	1	497.21	0.00	0.95	1.00
Caffeine	1	0.89	0.36	0.03	0.15
Stress	1	0.04	0.85	0.00	0.05
Caffeine*Stress	1	6.42	0.02	0.20	0.68
Error	26				
Total	30				
Corrected Total	29				

Table 8: ANOVA of Total Liquid Consumption (in mL) Time 6

Tests of Between-Subjects Effects TtlVol6 (Days 14 to 17)					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	3	2.24	0.11	0.21	0.50
Intercept	1	511.61	0.00	0.95	1.00
Caffeine	1	1.29	0.27	0.05	0.19
Stress	1	0.25	0.62	0.01	0.08
Caffeine*Stress	1	5.00	0.03	0.16	0.58
Error	26				
Total	30				
Corrected Total	29				

Table 9: Descriptives [Means (SDs)] of Daily Caffeine Consumption (mg)

	Days -6 to -1 (Time 1)	Days 0 to +2 (Time 2)	Days +3 to +6 (Time 3)	Days +7 to +9 (Time 4)	Days +10 to +13 (Time 5)	Days +14 to +17 (Time 6)
Control	0	0	0	0	0	0
Stress-only	0	0	0	0	0	0
*Caffeine- only	34.01 (17.12)	63.22 (37.93)	9.65 (21.57)	26.55 (34.89)	47.22 (43.46)	46.79 (31.38)
*Caffeine- Stress	23.16 (10.51)	71.12 (48.03)	26.51 (20.47)	25.07 (27.26)	33.85 (23.38)	44.59 (17.12)
* Does not include acute doses No shading indicates half-caffeine concentration in drinking water (1/2 g/L) Lighter shading indicates WSP at time of data collection Darker shading indicates WSP+SD at time of data collection						

Table 10: ANOVA of Caffeine Consumption (mg/kg) Time 1

Tests of Between-Subjects Effects Caff1 (Days -6 to -1) (does not include acute doses)					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	1	2.04	0.18	0.15	0.26
Intercept	1	56.68	0.00	0.83	1.00
Stress	1	2.04	0.18	0.15	0.26
Error	12				
Total	14				
Corrected Total	13				

Table 11: ANOVA of Caffeine Consumption (mg/kg) Time 2

Tests of Between-Subjects Effects Caff2 (Days 0 to 2) (does not include acute doses)					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	1	0.12	0.74	0.01	0.06
Intercept	1	33.73	0.00	0.74	1.00
Stress	1	0.12	0.74	0.01	0.06
Error	12				
Total	14				
Corrected Total	13				

Table 12: ANOVA of Caffeine Consumption (mg/kg) Time 3

Tests of Between-Subjects Effects Caff3 (Days 3 to 6) (does not include acute doses)					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	1	2.25	0.16	0.16	0.28
Intercept	1	10.35	0.01	0.46	0.84
Stress	1	2.25	0.16	0.16	0.28
Error	12				
Total	14				
Corrected Total	13				

Table 13: ANOVA of Caffeine Consumption (mg/kg) Time 4

Tests of Between-Subjects Effects Caff4 (Days 7 to 9) (does not include acute doses)					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	1	0.01	0.93	0.00	0.05
Intercept	1	9.52	0.01	0.44	0.81
Stress	1	0.01	0.93	0.00	0.05
Error	12				

Table 14: ANOVA of Caffeine Consumption (mg/kg) Time 5

Tests of Between-Subjects Effects Caff5 (Days 10 to 13) (does not include acute doses)					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	1	0.51	0.49	0.04	0.10
Intercept	1	18.89	0.00	0.61	0.98
Stress	1	0.51	0.49	0.04	0.10
Error	12				
Total	14				
Corrected Total	13				

Table 15: ANOVA of Caffeine Consumption (mg/kg) Time 6

Tests of Between-Subjects Effects Caff6 (Days 14 to 17) (does not include acute doses)					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Corrected Model	1	0.03	0.87	0.00	0.05
Intercept	1	45.75	0.00	0.79	1.00
Stress	1	0.03	0.87	0.00	0.05
Error	12				
Total	14				
Corrected Total	13				

Table 16: Descriptives of Horizontal Activity

Descriptive Statistics Horizontal Activity					
T1_OFA_HA	Caffeine	Stress	Mean	Std. Deviation	N
	Control	No Stress Control	9797.13	2855.27	8
		Stress	11874.38	2242.93	8
		Total	10835.75	2702.39	16
	Caffeine	No Stress Control	10757.14	4771.24	7
		Stress	8820.43	4858.03	7
		Total	9788.79	4733.83	14
	Total	No Stress Control	10245.13	3752.11	15
		Stress	10449.20	3888.05	15
		Total	10347.17	3755.67	30
T2_OFA_HA	Control	No Stress Control	8721.38	1098.26	8
		Stress	9643.88	2414.75	8
		Total	9182.63	1873.76	16
	Caffeine	No Stress Control	7048.14	1026.67	7
		Stress	5855.71	1774.70	7
		Total	6451.93	1524.12	14
	Total	No Stress Control	7940.53	1342.17	15
		Stress	7876.07	2844.65	15
		Total	7908.30	2185.68	30

Table 17: Overall rANCOVA for Horizontal activity

Within-Subject Effects					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Time	1	2.19	0.15	0.08	0.30
Time * BL_OFA_HA	1	0.20	0.66	0.01	0.07
Time * Caffeine	1	1.33	0.26	0.05	0.20
Time * Stress	1	0.06	0.81	0.002	0.06
Time * Caffeine * Stress	1	0.63	0.43	0.03	0.12
Error(Time)	25				

Between-Subjects Effects					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Intercept	1	13.47	0.001	0.35	0.94
BL_OFA_HA	1	10.03	0.004	0.29	0.86
Caffeine	1	2.91	0.10	0.10	0.38
Stress	1	0.45	0.51	0.02	0.10
Caffeine * Stress	1	2.25	0.15	0.08	0.30
Error	25				

Table 18: ANCOVA for Horizontal Activity at Time 2

Between-Subjects Effects T2 *HA ANCOVA (BL HA Covariate)					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Intercept	1	25.25	0.00	0.50	1.00
BL_OFA_HA	1	56.59	0.00	0.69	1.00
Caffeine	1	33.05	0.00	0.57	1.00
Stress	1	3.52	0.07	0.12	0.44
Caffeine * Stress	1	2.88	0.10	0.10	0.37
Error	25				

\*Time 2: After Warrior Stress Paradigm and Sleep Disruption

Table 19: Descriptives of Center Time/Movement Time

Descriptive Statistics Center Time/Movement Time					
	Caffeine	Stress	Mean	Std. Deviation	N
T1_OFA_ANX	Control	No Stress Control	0.14	0.07	8
		Stress	0.14	0.04	8
		Total	0.14	0.05	16
	Caffeine	No Stress Control	0.11	0.06	7
		Stress	0.14	0.08	7
		Total	0.13	0.07	14
	Total	No Stress Control	0.13	0.06	15
		Stress	0.14	0.06	15
		Total	0.14	0.06	30
T2_OFA_ANX	Control	No Stress Control	0.13	0.05	8
		Stress	0.13	0.03	8
		Total	0.13	0.04	16
	Caffeine	No Stress Control	0.13	0.05	7
		Stress	0.12	0.13	7
		Total	0.13	0.10	14
	Total	No Stress Control	0.13	0.05	15
		Stress	0.12	0.09	15
		Total	0.13	0.07	30

Table 20: Overall rANCOVA for Center Time/Movement Time

Within-Subject Effects					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Time	1	0.01	0.94	0.00	0.05
Time * BL_OFA_ANX	1	0.01	0.93	0.00	0.05
Time * Caffeine	1	0.34	0.57	0.01	0.09
Time * Stress	1	0.31	0.58	0.01	0.08
Time * Caffeine * Stress	1	0.25	0.62	0.01	0.08
Error(Time)	25				

Between-Subjects Effects					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Intercept	1	7.79	0.01	0.237	0.77
BL_OFA_ANX	1	2.58	0.12	0.094	0.34
Caffeine	1	0.00	0.95	0	0.05
Stress	1	0.10	0.76	0.004	0.06
Caffeine * Stress	1	0.05	0.82	0.002	0.06
Error	25				



Table 21: Descriptives of Vertical Activity

Descriptive Statistics Vertical Activity					
T1_OFA_VA	Caffeine	Stress	Mean	Std. Deviation	N
	Control	No Stress Control	1728.63	729.60	8
		Stress	1978.00	652.27	8
		Total	1853.31	680.84	16
	Caffeine	No Stress Control	1639.71	1010.61	7
		Stress	1158.71	372.40	7
		Total	1399.21	773.10	14
	Total	No Stress Control	1687.13	840.23	15
		Stress	1595.67	671.68	15
		Total	1641.40	748.85	30
T2_OFA_VA	Control	No Stress Control	1372.88	306.69	8
		Stress	1428.50	581.16	8
		Total	1400.69	449.82	16
	Caffeine	No Stress Control	1067.57	252.49	7
		Stress	868.43	278.26	7
		Total	968.00	275.38	14
	Total	No Stress Control	1230.40	314.97	15
		Stress	1167.13	534.51	15
		Total	1198.77	432.27	30

Overall Caffeine Estimates VA				
	Mean	Std. Error	Lower Bound	Upper Bound
Control	1629.63	118.38	1385.83	1873.44
Caffeine	1180.60	126.55	919.96	1441.24

Table 22: Overall rANCOVA for Vertical Activity

Within-Subject Effects					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Time	1	1.80	0.19	0.07	0.25
Time * BL_OFA_VA	1	0.01	0.92	0.00	0.05
Time * Caffeine	1	0.01	0.93	0.00	0.05
Time * Stress	1	0.03	0.86	0.001	0.05
Time * Caffeine * Stress	1	0.97	0.34	0.04	0.16
Error (Time)	25				

Between-Subjects Effects					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Intercept	1	17.52	0.00	0.41	0.98
BL_OFA_VA	1	2.00	0.17	0.07	0.27
Caffeine	1	6.71	0.02	0.21	0.70
Stress	1	0.38	0.55	0.02	0.09
Caffeine * Stress	1	2.12	0.16	0.08	0.29
Error	25				

Table 23: ANCOVA for Vertical Activity at Time 1

Between-Subjects Effects T1* VA ANCOVA					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Intercept	1	11.08	0.00	0.31	0.89
BL_OFA_VA	1	0.76	0.39	0.03	0.13
Caffeine	1	2.97	0.10	0.11	0.38
Stress	1	0.23	0.64	0.01	0.08
Caffeine * Stress	1	1.94	0.18	0.07	0.27
Error	25				

\*Time 1: After Warrior Stress Paradigm

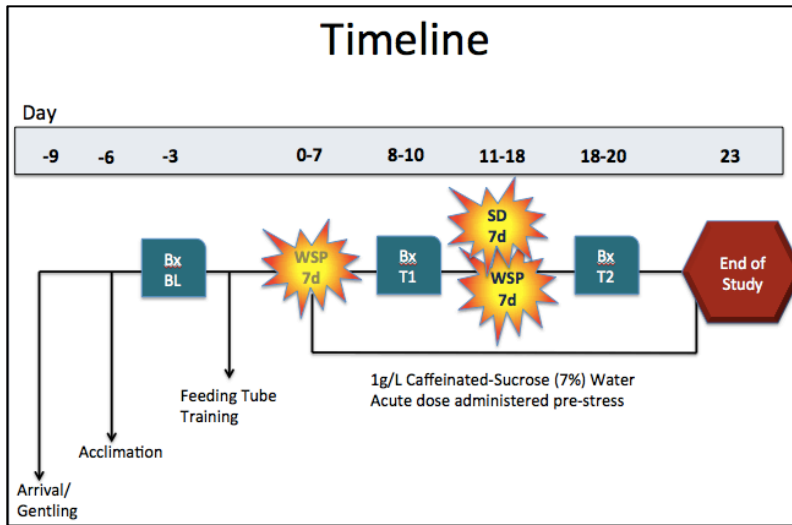
Table 24: ANCOVA for Vertical Activity at Time 2

Between-Subjects Effects T2* VA ANCOVA					
Source	df	F	Sig.	Partial Eta Squared	Observed Power
Intercept	1	17.56	0.00	0.41	0.98
BL_OFA_VA	1	3.65	0.07	0.13	0.45
Caffeine	1	10.63	0.00	0.30	0.88
Stress	1	0.40	0.53	0.02	0.09
Caffeine * Stress	1	0.99	0.33	0.04	0.16
Error	25				

\*Time 2: After Warrior Stress Paradigm and Sleep Disruption

## APPENDIX B: Figures

Figure 1: Experimental Timeline



Day 0 represents the initiation of the warrior stress paradigm, 1 g/L of caffeinated water in home cage water bottles, and administration of acute caffeine dose (60 mg/kg) via feeding syringe.

Bx BL (baseline behaviors); WSP (Warrior Stress Paradigm);  
 Bx T1 (behaviors time 1); SD (sleep disruption);  
 Bx T2 (behaviors time 2); 7d (7 days)

Figure 2: Warrior Stress Paradigm Schedule

Timeline of Stress Days		
Stress Day	Predator Stress	Unpredictable Event
1	Fox Urine (20 min)	None
2	Fox Urine (10 min)	Whistle at 12, 15 & 19 min
3	Fox Urine (10 min)	Coin Shake at 11, 14, & 17 min
4	Fox Urine (10 min)	Flashing Lights at 13, 16, & 19 min
5	Fox Urine (10 min)	Cage Shake at 12, 15, & 18 min
6	Fox Urine (10 min)	Flashing Lights at 12, 16, & 19 min
7	Fox Urine (10 min)	Whistle at 11, 13, 16 & 18 min
8	Fox Urine (10 min)	Coin Shake at 12, 16, & 19 min
9	Fox Urine (10 min)	Flashing Lights at 11, 15, 19 min
10	Fox Urine (10 min)	Cage Shake at 11, 14, & 17 min
11	Fox Urine (10 min)	Coin Shake at 13, 16, & 19 min
12	Fox Urine (10 min)	Whistle at 12, 14, 17 min
13	Fox Urine (10 min)	Flashing Lights at 11, 14, 18 min
14	Fox Urine (10 min)	Cage Shake at 12, 15, & 18 min

(4; 66; 106; 109)

Figure 3: Warrior Stress Paradigm Equipment



Photo by A.M. Yarnell, 2012

Figure 4: Acute Caffeine Feeding Syringe

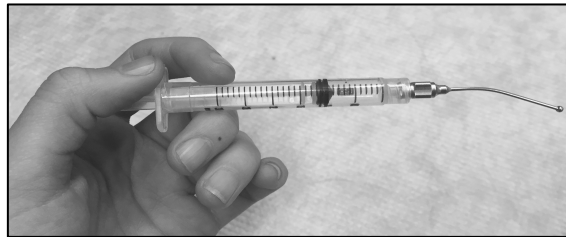


Photo by M.C. Baisley, 2015

Figure 5: Open Field Activity

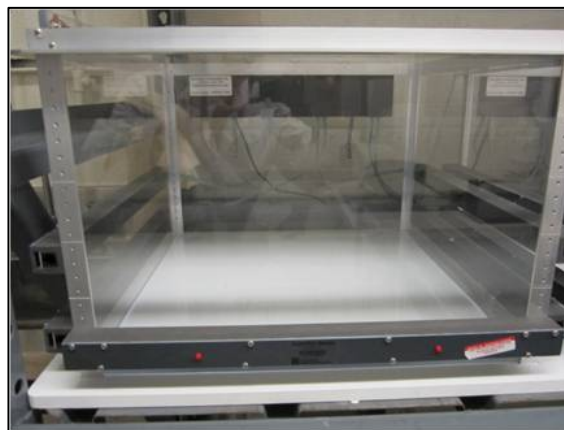
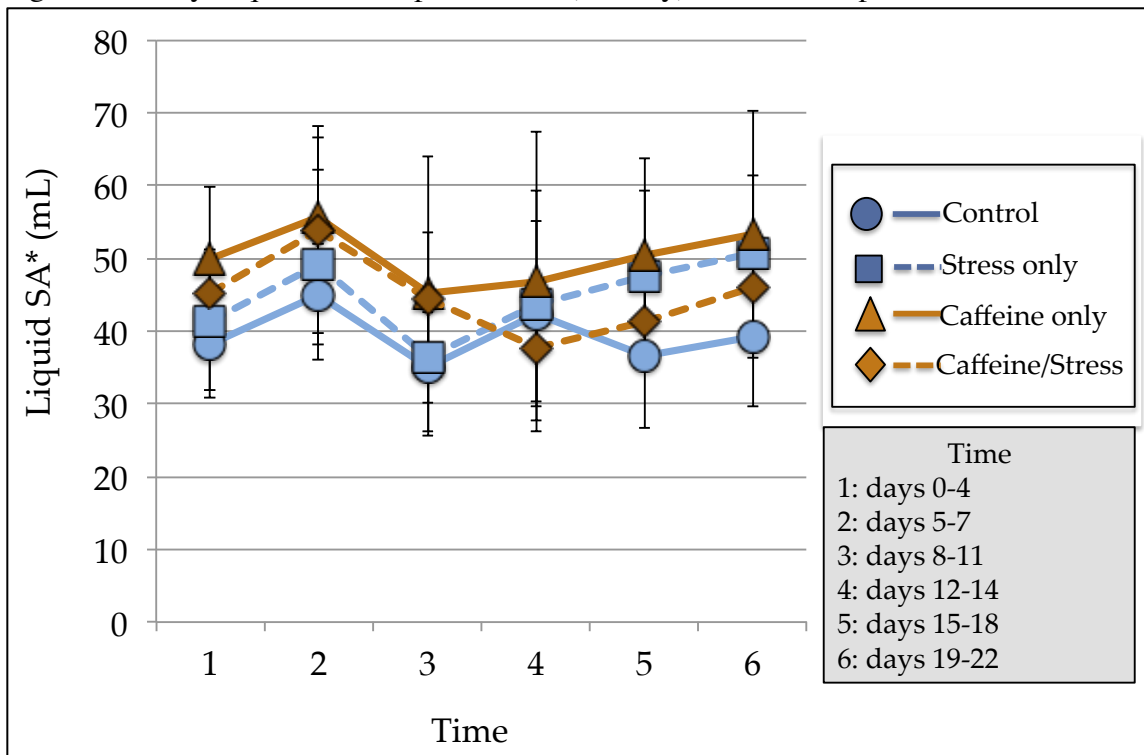


Photo by A.M. Yarnell, 2012

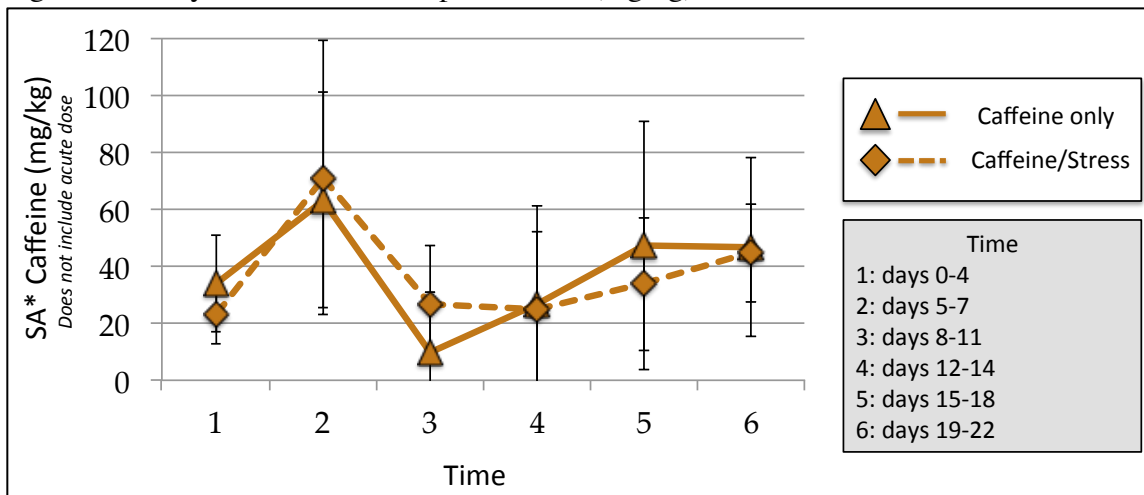
Figure 6: Daily Liquid Consumption Rates (mL/day) Across Groups



\* SA: self-administered

Error bars show  $\pm 1$  SD

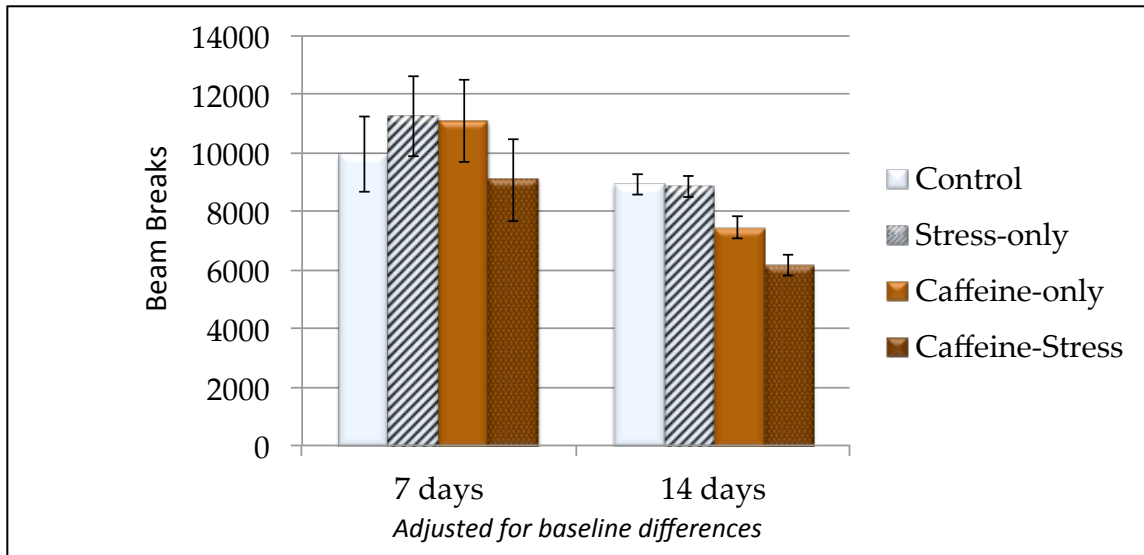
Figure 7: Daily Caffeine Consumption Rates (mg/kg) Across Time



\* SA: self-administered (home cage bottles)

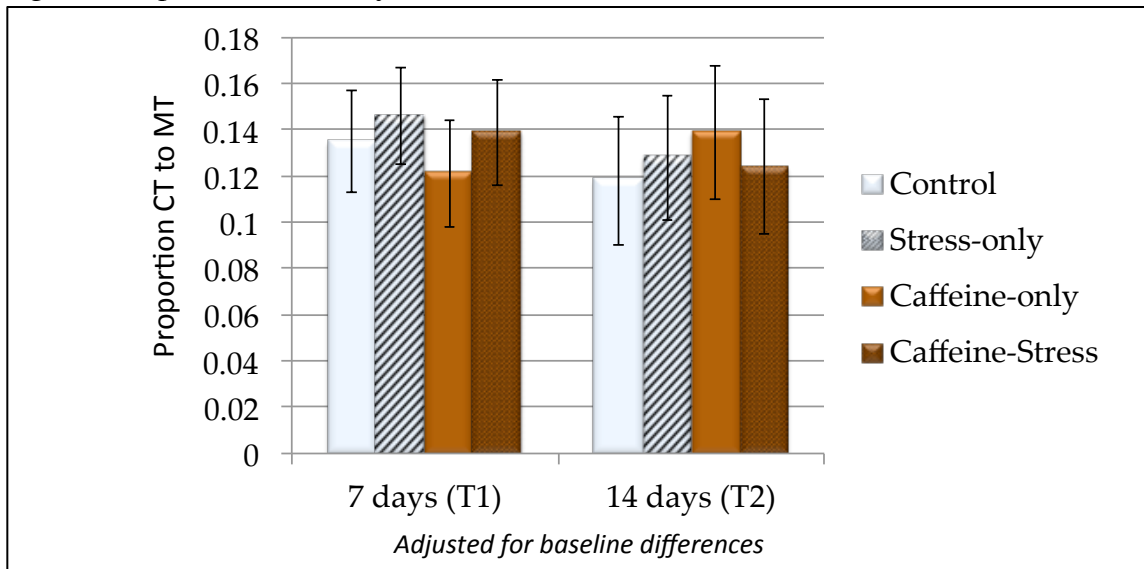
Error bars show  $\pm 1$  SD

Figure 8: Open Field Activity: Horizontal Activity



Error bars show  $\pm 1$  SEM

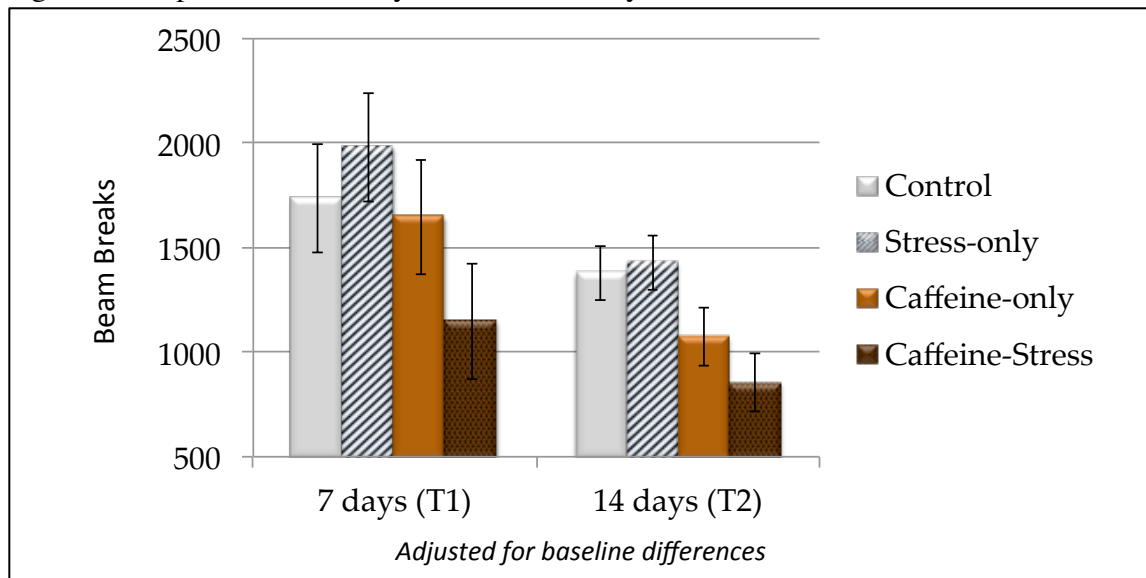
Figure 9: Open Field Activity: Center Time/Movement Time



CT (center time); MT (movement time)

Error bars show  $\pm 1$  SEM

Figure 10: Open Field Activity: Vertical Activity



Error bars show  $\pm 1$  SEM

## APPENDIX C: Administrative Documents



UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES

4301 JONES BRIDGE ROAD  
BETHESDA, MARYLAND 20814-4799



April 2, 2014

MEMORANDUM FOR DR. NEIL GRUNBERG, DEPARTMENT OF MEDICAL AND  
CLINICAL PSYCHOLOGY

SUBJECT: IACUC Approval of Protocol – Initial Review

The following application was reviewed and approved by the Uniformed Services University of the Health Sciences (USUHS) Institutional Animal Care and Use Committee (IACUC) via Designated Member Review on April 2, 2014:

Title of Application: "Behavioral investigations of nicotine and caffeine in rats (*Rattus norvegicus*)"

USUHS Protocol Number: MPS-14-898

Expiration Date: April 1, 2017


Supporting Grant(s) Number: E072194414

Name of Principal Investigator: Dr. Neil Grunberg

The USUHS has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare (OLAW), National Institutes of Health (NIH). The Assurance Number is A3448-01. The IACUC approved the above referenced application as submitted.

An annual review is required for each of the three years of this protocol. This review must be completed by the anniversary date of the protocol. If work is to be continued past the expiration date, a triennial review must be completed prior to the expiration date in order for work to be uninterrupted. Protocol expiration dates may not be extended, and no animal work may be done without an approved protocol. Although the IACUC may send reminders, it is the investigator's responsibility to submit an annual review form (Form 3206A) at least 30 days in advance, or a new Form 3206 for triennial review at least 60 days in advance of expiration.

Prior to placing your first animal order, please contact MAJ. Amanda Christy to schedule a pre-protocol planning meeting (295-3708). This meeting must occur to ensure animal numbers are loaded in the CART system and LAM resources are available to meet your needs.

  
Brian M. Cox, Ph.D.  
Chair, Institutional Animal  
Care and Use Committee

cc: Office of Research





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Brian M. Cox, Ph.D.  
Chair, Institutional Animal  
Care and Use Committee

cc: Office of Research

versus caffeine) factorial design will be used, with 3 animals per cell. Table 1 describes the planned dosing methods that will be used to compare the routes of administration and assess tolerance effects. The pilot animals will be housed for two weeks. Data from the elevated plus maze and open field activity will be collected at multiple time-points to observe behavioral effects of different caffeine administrations. These data will be used to make a final decision on which route of administration to use for the study. Time animals will be tested will not exceed 1 hour/day (only 1 behavior/day).

Table 1: Caffeine dosage plan for pilot animals

Route	Jell-O	Drinking water	Caffeine dosage
Acute dose	20 mg/kg Jell-O 30 min before behaviors	20 mg/kg syringe (oral) 30 min before behaviors	20 mg/kg in a 2 mL 5% aqueous sucrose solution
Chronic dose	2 x 40 mg/kg Jell-O per day	Ad libitum (1 g/L; avg 100 mg/kg/day)	80-100 mg/kg

(3) We would like to add the elevated plus maze (EPM; Pellow et al., 1985) as an additional behavioral measure. EPM is an established measure of anxiety-related behavior in rodents. The addition of this measure is consistent with the initial study aim to investigate the impact of caffeine on the stress response as demonstrated by various proxies of anxiety in rats. We would like additional measures of anxiety-related behavior to enhance validity of our study. EPM requires that the animals are recorded in the apparatus for five-minutes at each data-point. The EPM apparatus consists of two intersecting wooden bars, which create four arms. Two arms are closed in by walls and the other two arms are open. Anxiety-related behavior in EPM is demonstrated by the longer time spent in the enclosed spaces and less entries into the open arms. After the animals are recorded for five minutes in the apparatus, they are returned to their home cages. For the current study, we plan to collect EPM data across four time-points (two baseline measures & two post-stressor measures). The additional impact on the animals in terms of time and pain is negligible. This measure has been used previously in our lab for over ten years (e.g., Elliott et al., 2004; Elliott et al., 2005).

(4) We would like separate housing due to rats' highly sensitive olfactory senses and the influence opposite-sex pheromones may have on the behavior of both male and female rats (e.g., Zufall & Leinders-Zufall, 2007). We have concerns that this influence may confound our results.

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